Plant Molecular Biology 18: 47–53, 1992.
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A plant signal sequence enhances the secretion of bacterial ChiA in transgenic tobacco

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Received 19 July 1991; accepted in revised form 26 August 1991

Key words: chitinase, glycosylation, protein secretion, transgenic plants

Abstract

When the secreted bacterial protein ChiA is expressed in transgenic tobacco, a fraction of the protein is glycosylated and secreted from the plant cells; however most of the protein remains inside the cells. We tested whether the efficiency of secretion could be improved by replacing the bacterial signal sequence with a plant signal sequence. We found the signal sequence and the first two amino acids of the PR1b protein attached to the ChiA mature protein directs complete glycosylation and secretion of the ChiA from plant cells. Glycosylation of this protein is not required for its efficient secretion from plant cells.

Introduction

In eukaryotes most secreted proteins have been shown to possess a signal sequence of approximately thirty amino acids at the N-terminus, which when recognized by the appropriate cellular machinery leads to the translocation of the protein across the membrane of the endoplasmic reticulum [15]. Signal sequences show little homology at the amino acid level but do share common features including positive charge at the amino-terminus, an internal stretch of hydrophobic amino acids, and a polar carboxy-terminal region which contains the cleavage site [22]. These features are conserved in the eukaryotic and prokaryotic kingdoms with some signal sequences across kingdom boundaries [20, 24].

We have shown previously that when the *Serratia marcescens chiA* gene (which codes for a secreted protein, ChiA) is expressed in tobacco cells, a fraction of the expressed protein is modified by the attachment of complex glycans and secreted from plant cells [12]. In this paper, we describe experiments directed towards improving secretion of ChiA by plant cells. We have tested whether secretion depends upon the presence of a N-terminal signal sequence and if replacement of the signal sequence of ChiA with that of the tobacco PR1b protein increases secretion of the ChiA protein by plant cells. The secretion of mutated forms of ChiA lacking the consensus sequence for N-linked glycosylation was also investigated.

Materials and methods

Plasmid construction

The pChiA plant transformation series derivatives were all prepared in the binary plasmid pJJ2964. This plasmid contains T-DNA carrying an *nptII* gene driven by the *nos* promoter (to enable selection of transformed tissue on kanamycin), and unique *Bam* HI and *Hin*d III cloning sites. Manipulations on the *chiA* gene were carried out with it cloned in the vector pUC118 as a fragment containing the cauliflower mosaic virus (CaMV) 35S promoter followed by a leader from the petunia *Cab22L* gene [6], upstream from the complete *chiA* gene from *Serratia marcescens*. Downstream from the *chiA* gene is a fragment carrying the polyadenylation signals from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene. The *chiA* gene had the following modifications to its sequence [7]: (1) a novel *Nco* I site at position + 1; (2) a novel *Sma* I site at position 78; (3) the *Sma* I site present in the native sequence at position 951 has been removed. Oligonucleotide-directed mutagenesis was used to make all these changes. The novel *Nco* I site changes the second amino acid in the signal peptide from Arg to Ala; the other changes have no effect on the protein sequence.

To construct the plasmid pChiA, the *Bgl* II-*Hin*d III fragment from the pUC118 derivative described above was ligated into *Bam* HI-*Hin*d III-cut pJJ2964. The plasmid pChiA-M was constructed following oligonucleotide loop-out mutagenesis of the *chiA* gene cloned in pUC118, which removed all the codons of the ChiA signal sequence (amino acids 2 to 23) except for the initiator methionine. The *Bgl* II-*Hin*d III fragment carrying the modified *chiA* gene and the plant expression signals was then ligated into pJJ2964 as described for pChiA to form pChiA-M.

The plasmid pPRSSChiA was constructed by synthesizing the codons for the PR1b signal sequence plus the first two amino acids of the mature PR1b protein as two complementary oligonucleotides, with a half *Nco* I site at the 5′ end and a half *Sma* I site at the 3′ end. This was ligated into the *Nco* I and *Sma* I sites at the 5′ end of the *chiA* gene. pPRSSChiA was then constructed by ligating the *Bgl* II-*Hin*d III fragment into pJJ2964, as described above for the other pChiA plasmids.

To remove the glycosylation sites from the ChiA protein, the codons for amino acids at potential N-glycosylation sites (Asn-X-Ser/Thr) were identified on the DNA sequence, then oligonucleotide-directed mutagenesis was used to change the codon for the third amino acid in each site to alanine. All manipulations were carried out on the *chiA* gene cloned in pUC118 and all changes were verified by DNA sequence analysis. The plasmids pChiA-G and pPRSSChiA-G were then constructed; these are identical to pChiA and pPRSSChiA except that both contain all four of the site-directed mutations that remove the four consensus glycosylation sites.

Plant cell tissue culture

Plant transformations, establishment, maintenance and sampling of suspension cultures, and protoplast preparations were as described [12]. All plant transformations were carried out using *Nicotiana tabacum* cv. SR1.

Protein extraction and measurement

Protein extractions, electrophoresis and immunoblotting of protein extracts were all carried out as described [12], except that immunoblots were developed using an alkaline phosphatase conjugate in place of the horseradish peroxidase conjugate. The buffer used for making protein extracts for gel and enzyme analysis contained 84 mM sodium citrate, 32 mM sodium phosphate, 6 mM ascorbic acid, and 14 mM β-mercaptoethanol, pH 5.5.

Nucleic acid analysis

DNA manipulations were carried out as described [13] or according to enzyme suppliers'

ıstructions. RNA extraction from leaf tissue and rimer extension analysis for the quantification of :eady-state RNA and confirmation of transcription start sites was carried out as described [6].)ligonucleotide-directed mutagenesis was by the ıethod of Kunkel [11]; all changes were conrmed by DNA sequence analysis as described y Sanger *et al.* [17]. Oligonucleotide primers for ıutagenesis and sequence analysis were made on n Applied Biosystems 381A DNA synthesizer.

Results

To test whether the bacterial signal sequence of ChiA is required for plant cell secretion, we prepared a deletion mutant of the *chiA* gene lacking he region which specifies the codons of the signal sequence, pChiA-M (amino acids 2 to 23); the amino terminal of the resulting protein from pChiA-M is shown in Fig. 1. The ChiA protein was then expressed in plant cells with and without its signal sequence by transformation with the binary plasmids pChiA and pChiA-M. In parallel, to determine whether the fraction of ChiA secreted by tobacco cells could be increased by fusion to the signal sequence from a secreted plant protein, we constructed a translational fusion between PR1b and the mature ChiA protein. We chose the tobacco PR1b protein as the source of a plant signal sequence because complete sequence information was available for the PR1b gene and the extracellular location of the protein has been well studied. The portion of the *chiA* gene encoding the signal sequence of ChiA was replaced with that encoding the signal sequence from PR1b so that the resulting fusion protein contains the PR1b signal sequence plus the first two amino acids of the PR1b mature protein (Gln-Asn) in place of the first two amino acids of the mature ChiA protein (Ala-Ala) (see Fig. 1). This fusion protein was also expressed in plant cells by transformation with the binary plasmid pPRSSChiA. At least 10 independent transformants were prepared for each of the constructions pChiA, pChiA-M and pPRSSChiA, then 2 plants from each group were selected for subsequent comparative analyses. The representative plants were chosen so that the transformants carrying the different *chiA* genes each were expressing similar steady-state *chiA* mRNA levels and ChiA protein.

Immunoblots of leaf proteins isolated from two plants transformed with pChiA show multiple bands (Fig. 2, lanes 2 and 3), the most prominent of which comigrates with ChiA protein expressed in *Escherichia coli* (Fig. 2, lane 1). We have previously shown that the most prominent species is an intracellular form. and the fainter bands of higher molecular weight are glycosylated, secreted forms of ChiA [12]. Immunoblots of protein from two plants transformed with pChiA-M (Fig. 2, lanes 6 and 7) show a single band which comigrates with ChiA from *E. coli*; no species of higher molecular weight can be detected, which indicates that glycosylation of ChiA does not occur when it is expressed without a signal sequence. Immunoblot analysis of leaf extracts from plants transformed with pPRSSChiA shows that, in contrast to those transformed with pChiA, all of the cross-

pChiA MAKFNKPLLA LLIGSTLCSA AQA↓APGKPT

pChiA-M MAAPGKPT

pPRSSChiA MGFLLFSQMP SFFLVSTLLL FLIISHSSHA ↓QNPGKPT

Fig. 1. Deduced amino acid sequences of the deletion and fusion derivatives of the *chiA* gene. The sequences in each case extend to the 7th amino acid in the mature ChiA sequence. The vertical arrow indicates the likely site of cleavage of the signal peptide (known for ChiA in *E. coli* and predicted for PRSS on the basis of the known cleavage point in the PR1b protein).

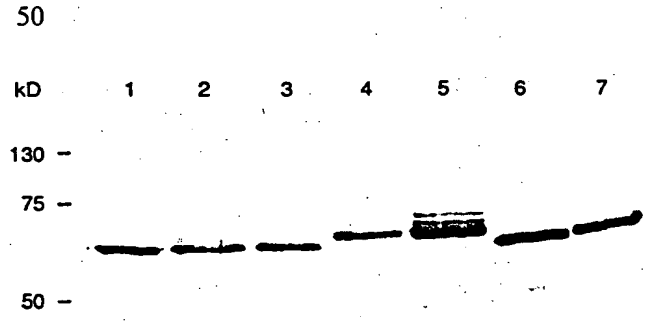

Fig. 2. Immunoblot with ChiA antibody to the total leaf protein (100 μg) from individual tobacco plants transformed with ChiA derivatives. Lane 1: ChiA from *E. coli* (200 ng); lanes 2 and 3: pChiA (ChiA signal sequence): lanes 4 of 5: pPRSS-ChiA (PR1 signal sequence): lanes 6 and 7; pChiA-M (no signal sequence).

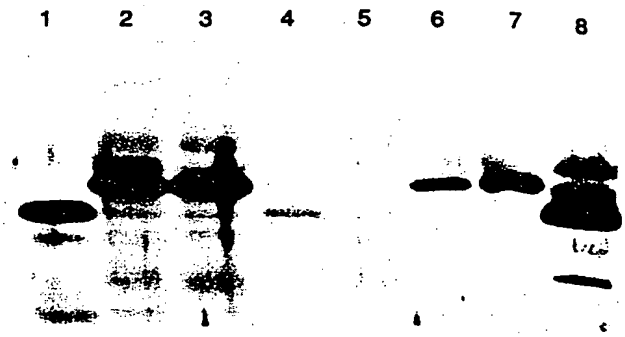

Fig. 3. Immunoblot with ChiA antibody to protein isolated from suspension culture medium. Lane 1: ChiA protein from *E. coli* (200 ng); lanes 2 and 3: medium from pPRSSChiA cells; lanes 4 and 5: medium from pChiA-M cells; lanes 6 and 7: medium from pChiA cells (lanes 2–7 each contain protein extracted from 1 ml culture medium); lane 8: 100 μg leaf protein from ChiA plant.

reacting protein is in a position corresponding to the glycosylated forms of ChiA (Fig. 2, lanes 4 and 5).

We assayed the level of secretion of ChiA from these different transgenic plant cells by analyzing the media from plant cell suspension cultures and by comparing the profiles of protein extracts from leaf protoplasts and corresponding whole leaves. We have shown that these approaches give consistent results and correctly demonstrate secretion of the PR1b secreted tobacco protein (unpublished data). The culture fluid from suspension cultures established from individual plants transformed with pChiA, pChiA-M or pPRSSChiaA, was analyzed by immunoblotting (Fig. 3). There is little or no ChiA protein in the medium from the pChiA-M transformed cells (lanes 4 and 5), and high levels of ChiA in the medium from the pPRSSChiA or pChiA transformed cells (lanes 2, 3, 6 and 7). Furthermore the ChiA which is present in the culture medium from pPRSSChiA and pChiA transformants is the higher-molecular-weight glycosylated form. The faint band seen in lanes 4 and 5, which comigrates with the bacterial standard (lane 1), probably corresponds to non-glycosylated non-secreted ChiA which is in the culture fluid as a consequence of cell death. These data from the analysis of suspension culture media suggest that in the absence of any signal sequence (pChiA-M) the ChiA which is expressed is not secreted. In the presence of a signal sequence, either the ChiA signal (pChiA), or the PR1b signal (pPRSSChiA), ChiA protein is glycosylated and secreted. The observation that higher levels of ChiA protein appear in the medium from pPRSSChiA transformants (Fig. 3, lanes 2, 3) than from pChiA transformants (Fig. 3, lanes 6, 7) suggests that secretion is more efficient when the PR1b signal is fused to ChiA.

Since secreted proteins will be present in leaf tissues extracts but absent from washed protoplasts, we have compared these tissues from the transgenic plants to further determine whether secretion is occurring. The results from typical experiments comparing these two tissues are shown in Fig. 4. In the total leaf extract from a plant transformed with pCHiA (lane 7), different molecular weight forms of the ChiA protein can be seen which correspond to glycosylated (upper) and non-glycosylated (lower) forms of the protein. In washed protoplasts of these plants, only the non-glycosylated (lower) form of ChiA is seen (lane 6), which is consistent with our above result indicating that the glycosylated higher-molecular-weight forms are secreted from plant cells. In plants transformed with pChiA-M, the protein profiles of washed protoplasts (lane 2) and total leaf extract (lane 3) are identical and correspond to the *E. coli* standard (lane 1), indicating that

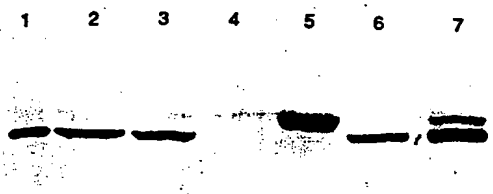

Fig. 4. Immunoblot of protein from leaf or from washed protoplasts. Lane 1: ChiA from *E. coli* (200 ng); lanes 2 and 3: pChiA-M; lanes 4 and 5: pPRSSChiA; lanes 6 and 7: pChiA. Lanes 2, 4 and 6 are from protoplasts; lanes 3, 5 and 7 are from leaf. Lanes 2 to 7 each contain 100 μg total protein.

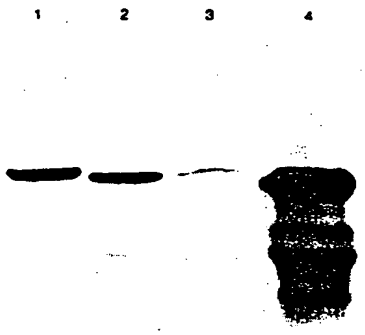

Fig. 5. Immunoblot of protein from protoplast and leaf extracts from plants expressing ChiA lacking glycosylation sites. Lane 1: *E. coli* ChiA (150 ng); lanes 2 and 3: pChiA-G; lanes 4 and 5: pPRSSChiA-G. Lanes 2 to 5 each contain 50 μg total protein; lanes 2 and 4 are leaf extracts, lanes 3 and 5 are protoplast extracts.

glycosylation has not taken place and that little or no secretion is occurring. The profiles from washed protoplasts and total leaf extracts of pPRSSChiA transformants are shown in lanes 4 and 5, and in this case the ChiA protein is present solely as a higher-molecular-weight form, none of which is detected inside washed protoplasts. These data comparing proteins from protoplasts and total leaf extracts confirm that secreted and glycosylated forms of ChiA protein occur only if a signal sequence is attached, and if the PR1b signal sequence is used then all of the ChiA protein is secreted and glycosylated.

There are four consensus N-glycosylation sites (Asn-X-Ser/Thr) in the predicted ChiA protein sequence. We constructed a derivative of the *chiA* gene where all four sites were 'inactivated' by altering the last codon in the consensus site to Ala. To express the mutant ChiA proteins in plant cells, the binary plasmids pChiA-G and pPRSSChiA-G were used to produce transformed tobacco plants. RNA and protein analysis was used to identify plants expressing high levels of the mutant ChiA proteins. (We noted that expression at both the RNA and protein level was generally significantly higher for plants transformed with pPRSSChiA-G than pChiA-G.)

We compared the protein profile in washed protoplasts with that in total leaf extracts from pChiA-G and pPRSSChiA-G transformants (Fig. 5). The ChiA protein in all plant extracts co-migrated with the mature ChiA protein as purified from bacteria, as would be predicted if no glycosylation were occurring. In pChiA-G transformants, the intensity of the ChiA band was greater in total leaf extract (lane 2) than in protoplast extracts (lane 3), suggesting that, as with the wild-type protein, secretion of ChiA does occur but not all of the ChiA is being secreted from the cells. In pPRSSChiA-G transformants, a trace of ChiA was detected in the protoplast extracts (lane 5) in contrast to the very high levels in total extract from leaf (lane 4), indicating that most or all of the unglycosylated ChiA protein is being secreted.

Discussion

We have investigated the secretion of the bacterial ChiA protein from plant cells. We had previously demonstrated that ChiA fused to the bacterial signal sequence is inefficiently secreted by plant cells. Here we show that the ChiA protein is fully secreted when the signal sequence derived from the tobacco PR1b protein is fused to the ChiA mature protein and secretion does not occur in the absence of a signal sequence. The lack of secretion in the absence of a signal sequence is expected, given the role of signal sequences in mediating targeting of proteins to the lumen of the endoplasmic reticulum in eukaryotic cells [16].

The fact that no detectable glycosylation of ChiA occurs in the absence of a signal sequence is also expected, since the initial transfer of glycans to proteins occurs as the proteins cross the ER membrane [9].

The most likely explanation for the improved efficiency of ChiA secretion in pPRSSChiA transformants is that the possession of a plant signal sequence improves the ability of the ChiA protein to enter the secretory pathway of the plant cells in which it is expressed. An alternative explanation is that the mature ChiA peptides arising from pChiA- and pPRSSChiA-transformed plants differ in the two N-terminal amino acids, and this difference could alter the mature protein so that it would behave differently in the plant secretory pathway. While this explanation cannot be ruled out, we believe it to be less likely, as we have not detected any significant differences in the physical or enzymological properties of the ChiA expressed with a bacterial or a plant signal sequence. (The precise point of cleavage of the signal sequences when expressed in plant cells remains to be determined.)

Signal sequences show considerable degeneracy, so that even random peptide sequences can function as signal sequences [8]; however, significant differences between prokaryotic and eukaryotic signal sequences are revealed when large numbers of sequences are analyzed statistically [23]. These differences may be reflected in the ability of signal sequences to function efficiently in heterologous hosts. There are reports where the use of a signal sequence native to the organism in which the protein is being expressed can enhance the secretion of a heterologous protein [1, 2, 19], and others where more efficient secretion of a foreign protein is seen when it possesses its own signal sequence rather than one derived from the organism in which it is expressed [2, 19]. Determining which features of the PR1b N-terminus are relevant in mediating the efficient secretion of ChiA from plant cells would be an interesting area for further study.

The fact that ChiA is apparently completely located outside the cell when expressed with a plant signal sequence may be taken as further evidence that the pathway for secretion in plant cells is a default pathway, requiring no positive sorting information other than the possession of a functional signal sequence. Thus it seems likely that many other proteins could also be engineered to be plant secretory proteins. In support of this, Denecke *et al.* [3] have recently shown that three normally cytoplasmic proteins can be secreted from plant cells by the attachment of a suitable signal sequence.

Glycan side-chains attached to proteins probably have multiple roles [14]; it has often been observed that prevention of glycosylation also prevents the secretion of the altered protein. Sometimes this can be attributed to decreased stability of the altered protein to proteases [4, 14], or to aggregation of the protein [5]. There are also cases where the non-glycosylated forms of the protein are secreted as efficiently as are the glycosylated forms [10, 18]. Thus the role of glycans in intracellular targeting is not simple and cannot be generalized. The likelihood of a direct role for glycan residues in some aspect of protein targeting in eukaryotic cells (for example, by interacting with a receptor as opposed to simply changing the physical properties of the protein) seems remote. Only in the case of lysosome is targeting mediated by mannose-6-phosphate residues [21]. The results presented in this paper demonstrate clearly that the efficiency with which ChiA can be secreted by plant cells is not influenced by the extent to which it is glycosylated.

Acknowledgements

We thank Eva Penzes, Paul Zankowski and Jeff Townsend for plant transformations; Rino Lee for expert technical assistance. This research was partly funded by NSF-SBIR Grant ISI-8701346.

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